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A Single-Molecule Study to Resolve How Kinases Prevent Chromosomal Mis-Segregation
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Mitosis is an exquisitely choreographed process that relies on specialized interactions between kinetochores on chromosomes and the microtubules of the mitotic spindle. The process is orchestrated by an error-correction system that, remarkably, detects improperly aligned chromosomes by sensing a lack of tension in their kinetochore-microtubule attachments. This surveillance system ensures that each daughter cell receives exactly one copy of each chromosome; failure leads to cancer and birth defects. Two surveillance system components, the Mps1 and Ipl1 protein kinases, are of particular interest because indirect evidence suggests that they are responsible for core system functions. Direct tests of their functions have been lacking because the tension that they are thought to sense cannot be accurately measured or controlled in a cell. Very recently, our group completed the first-ever study of kinetochore-microtubule attachments reconstituted in vitro, positioning me to study the tension-sensing surveillance system in ways never before possible. I will apply precise forces to kinetochore-microtubule attachments using an optical trap, and I will make genetic changes to the kinetochores at sites where the kinases are thought to act. With these powerful single-molecule techniques, I will uncover how Mps1 and Ipl1 (1) modify kinetochores to destabilize their microtubule attachments, and (2) operate on unattached, improperly attached, and properly attached kinetochores to promote the formation of proper attachments. Ultimately, my work will guide efforts currently underway to develop new chemotherapeutic inhibitors of Ipl1 and Mps1. It will also help us to understand how cells sense and respond to force at the molecular level in other contexts.

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Insights into the Micromechanical Properties of the Metaphase Spindle
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During cell division, the microtubule-based metaphase spindle is subjected to mechanical forces that act in diverse orientations and over a wide-range of timescales. Currently, we cannot explain how this micron-sized, dynamic cytoskeletal structure generates and responds to forces while maintaining overall stability, as we have a poor understanding of its micromechanical properties. Here we combine the use of force-calibrated needles, high-resolution microscopy, and biochemical perturbations to analyze the vertebrate metaphase spindle's timescale- and orientation-dependent viscoelastic properties. We find that the metaphase spindle is mechanically anisotropic, and deforms either elastically or viscously depending on the timescale of applied force. We also find that spindle viscosity depends on the dynamics of microtubule crosslinking and the density of the filament. Spindle elasticity can be linked to the rigidity of kinetochore and non-kinetochore microtubules, which have different polymerization dynamics and stability, and also to spindle pole organization by kinesin-5 and dynein. These data suggest a quantitative model for the micromechanics of this cytoskeletal architecture and provide insight into how structural and functional stability is maintained in the face of different forces, such as those that control spindle size and position, and can result from deformations associated with chromosome movement.

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Symmetrical Shape of the Meiotic Spindle is Dynamically Balanced
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Meiotic spindle assembly is critical for achieving accurate chromosome segregation. The spindle mainly consists of microtubules and molecular motors. Recent studies have suggested that bipolar spindle formation requires the force balance sustained by molecular motors and polymerization-depolymerization dynamics of microtubules. However, it is not well understood whether and how the two pole structures are symmetrically balanced in a spindle. In this study, we quantitatively measured the mechanical stiffness, the microtubule density, and the response to the deformation of spindle poles by micromanipulation techniques and 3D analysis. To deform the spindle self-assembled in *Xenopus* egg extract, we inserted two glass micro-needles into a pole region at one side and widened it perpendicularly to the pole-to-pole axis. We found that the stiffness and the microtubule density in the manipulated side of pole region reduced upon widening. Unexpectedly, the reduction was also observed after a while in the unmanipulated side, which resulted in the formation of a symmetrical defocused barrel-like shape. On the other hand, it has been reported that inhibition of the dynein function by the addition of dynein-dynactin inhibitor causes the defocusing of pole regions, such that the barrel-shaped structure is formed. We compressed one side of the barrel-shaped spindle using a pair of glass micro-needles and found that the stiffness and the microtubule density in the compressed region increased. These changes also occurred in the unmanipulated side, which was accompanied by the bipolar spindle formation. Our results suggest that symmetrical shape of the spindle is dynamically balanced for proper cell division.

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Tension-Dependent Dynamic Microtubule Model for Metaphase and Anaphase Phenomena
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We present a theoretical model describing metaphase chromosome oscillations, microtubule (MT) attachment error correction, and anaphase chromosome separation. During metaphase, chromosome pairs align near the center of a bipolar MT spindle and oscillate as the MTs attaching them to the cell poles polymerize and depolymerize. Simultaneously, the cell fixes misaligned chromosome pairs by some tension-dependent mechanism. In anaphase, chromosome pairs separate as depolymerizing MTs pull each chromosome toward its respective cell pole. Instead of including all known components to develop a comprehensive, species-specific description, we introduce a minimal model based on fundamental properties of MT kinetics. We use the tension-dependence of single MT polymerization/depolymerization kinetics measured by Akiyoshi et al. [1] and assume the same functional dependence for compressed MTs. We apply these to a many MT model, and solve this stochastic model numerically and by a master equation approach. We find that the tension dependence of rates enhances the speed of single chromosome pulling by MTs during anaphase- or error-correction-like behavior. Additionally, the force-velocity curve for a single chromosome attached to dynamic MTs exhibits bi-stability: at high loads, large tension fluctuations induce MTs to spontaneously switch from a depolymerizing state into a polymerizing state. The system is hysteretic; to recover depolymerization from the polymerizing state, the load must be decreased to a far smaller value than that required to initially induce polymerization. This behavior leads to the chromosome oscillations we observe in the two-chromosome system. Interestingly, we observe breathing oscillations, which are not captured by any other chromosome oscillation model. Our minimal model reflects general features of the underlying mechanisms of these phenomena, and reveals how different components control chromosome dynamics through the rate constants.
[1] Akiyoshi et al. (2010) Nature 468, 576-579.

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Phosphoregulation of the Ndc80 and Dam1 Subcomplexes Promotes Release of Kinetochores from Dynamic Microtubules via Multiple Mechanisms
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During mitosis, multiprotein complexes called kinetochores orchestrate chromosome segregation by forming load-bearing attachments to dynamic microtubule tips, and by participating in phosphoregulatory error correction. Aurora B kinase phosphorylates the major microtubule binding subcomplexes, Ndc80 and (in yeast) Dam1, to promote release of erroneous attachments, giving another chance for proper attachments to form. It is unknown whether Aurora phosphorylation promotes release directly, by increasing the rate of kinetochore detachment, or indirectly, by destabilizing the microtubule tip. Moreover, the relative importance of phosphorylation of Ndc80 versus Dam1 in the context of whole kinetochores is unclear. To address these uncertainties, we isolated native yeast kinetochore particles carrying phosphomimetic mutations on Ndc80 and Dam1, and applied advanced laser trapping techniques to measure the strength and stability of their attachments to individual dynamic microtubule tips. Composition of the purified particles was unaffected by the phosphomimetic mutations, suggesting that phosphorylation at these sites does not disrupt kinetochore structure. Rupture forces were reduced by phosphomimetics on both subcomplexes, in an additive manner, indicating that both subcomplexes make independent contributions to attachment strength. Likewise, phosphomimetics on either subcomplex reduced attachment lifetimes under constant force, primarily by accelerating detachment during microtubule growth. Phosphomimetics on Dam1 also increased the likelihood of switches from microtubule growth into shortening, further promoting release

in an indirect manner. Together our results suggest that, in vivo, Aurora B releases kinetochores via at least two mechanisms - by directly weakening the kinetochore-microtubule interface, and also by destabilizing kinetochore attached microtubule tips.

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Kinesin-14 Ncd Microtubule Rotational Motility: A Mathematical Model

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The kinesin-14 motor Ncd steps off-axis along the microtubule longitudinal axis as it moves towards the minus end, causing microtubules moving on ensembles of Ncd motors bound to a glass surface to rotate as they glide. Based on crystal structures, we propose that the rotations arise from a structural asymmetry of the motor when it binds to a microtubule. We have derived a mathematical model to explain the dependence of the rotations on ATP concentration by estimating the probability of off-axis steps by the motor using previously published motility data and modeling the relationship between ATP concentration and the number of motors bound to a microtubule using available kinetic data. Unlike previous models, our model not only takes into account the motor structure and kinetics, but also the effects of multiple motors bound to the same microtubule. While our model is specific for Ncd, it provides a mathematical explanation of how nonprocessive kinesin motors, like Ncd, function in ensembles to induce the microtubule translocation and sliding required for motor function in spindle assembly and elongation.

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Microtubule Doublet Curvature and its Role in Cilia Actuation

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Motile cilia are hair-like organelles that beat to produce fluid flow. It is not fully understood how these incredibly complex molecular machines function at the protein level to produce complicated beat patterns. The mechanical properties of microtubule doublets, the main structural element of the cilium, have not been well studied. Here we show that doublet microtubules separated from human airway cilia curl up with an average radius of curvature of 1.39 +- 0.01 μm, determined from transmission electron and atomic force imaging. This result is surprising given that cytoplasmic microtubules have a persistence length on the order of millimeters, but is consistent with curvature seen in the literature. Doublet microtubules were isolated by digesting the axoneme with trypsin and inducing sliding by the addition of ATP. After sliding, transmission electron images showed that dynein motors were still attached, which is consistent with prior studies. We prepared samples with a fraction of the outer dynein arms removed using a 0.6M potassium chloride salt extraction. Removal of dynein was confirmed by SDS-PAGE. Samples with removed dynein had an increased radius of curvature, 2.3 +- 0.1 μm. The straightening of the doublet microtubules with the removal of dynein motors implies that the asymmetric distribution of dynein motors causes a stress that bends the doublet microtubule. Our results suggest an alternative source for curvature in the beating cilium. The distribution of dynein heads bound to a microtubule doublet is known to change dynamically during the cilium beat. Changing distribution of dynein heads bound to the microtubules could induce changes in the curvature of the cilium without the need for the motors to apply a shear force.

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The Mechanical Mechanism of Platelet Induced Clot Stiffening

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The mechanical properties of blood clots are important to properly stem the flow of blood at the site of vascular injury. A blood clot is a complex material composed of many different proteins and cells. However, particularly important for its mechanical properties, is a meshwork of the protein fibrin which serves as the structural scaffold of the clot. One important feature of fibrin network mechanics is strain-stiffening: a stiffness that is constant at low strains and increases non-linearly with strain at high strains. During blood coagulation, fibrin forms in the presence of platelets which are known to greatly influence the mechanics and structure of fibrin gels. Although both platelets and fibrin are very important to the mechanics of blood clots, the underlying principles that determine fibrin mechanics and how platelets alter these remains poorly understood. In our study, we investigate the origin of strain-stiffening in fibrin gels and how this is influenced by platelets. Using confocal microscopy subsequent image analysis, we track the 3D network structure as it undergoes shear. We find that the mechanics of the network are dictated largely by its structure. Specifically, at low strains the network utilizes soft bending modes to deform without stretching the individual fibers, while at high strains these modes are exhausted and the fibers must begin to become stretched. To understand how platelets change these properties, we polymerize fibrin gels in the presence of activated platelets. We then image the structure of the network and measure its corresponding mechanical changes. We find that the low-strain stiffness increases with increasing platelet concentration while the high-strain stiffness remains unaltered. Platelets induce aster-like structures in the fibrin gel. The altered mechanical and structural properties are consistent with platelets reducing the number of available soft bending modes.

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Nanopost Arrays Indicate that GPIb-VWf Interactions Play a Role in Platelet Contractility Independent of Integrin αIIbβ3

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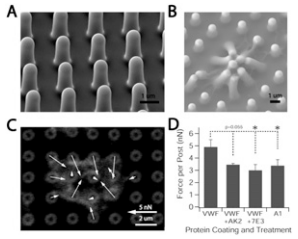
The first step of hemostasis involves recognition of von Willebrand factor (VWF) by platelets. These cells bind to domains of VWF using GPIb-IV-X and αIIbβ3 receptors. Because platelets must contract to shrink and stabilize the hemostatic clot, we investigated the contribution of each of these receptors in supporting platelet forces. For this, we developed a new tool using E-beam lithography to fabricate nanoposts for force measurements (Fig A). VWF was adsorbed to the tips of these posts to allow for platelet adhesion (Fig B). Nanoposts were fluorescently labeled to track deflections created by individual platelets (Fig C). Our results show that platelets treated with antibodies 7E3 and AK2, which inhibit αIIbβ3 and GPIb respectively, exerted significantly lower forces. To confirm that GPIb-VWF interaction can support platelet contraction, we used the recombinant GPIb-IX-V binding region of VWF (A1 domain) (Fig D). Platelets were unable to generate force on A1 domain in the presence of soluble GPIb, or using bovine serum albumin and Pluronic F-127 as a substrate. Together, these results indicate that platelets are able to transmit cytoskeletal contractile forces in an integrin-independent manner.



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Occlusion of Micro-Capillaries by Malaria Infected Red Blood Cells

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Malaria-infected red blood cells (iRBCs) can easily occlude micro-capillaries because of their anomalous stiffness and stickiness compared with health red cells. Previous work suggested three factors in the loss of deformability of iRBCs: (i) the stiffening of the membrane, (ii) the reduction of the cell's surface/volume (S/V) ratio, and (iii) the presence of solid parasites inside the cell. These factors have been examined in experiments and simulations of the stretching of iRBCs by optical tweezers, but not in capillary flows. In this work, we investigate the influence of the three factors on the blockage of micro-channels by using the smoothed particle hydrodynamic method. Within the parameter range tested, our results indicate the solid parasites as the main agent for micro-capillary occlusion. The decrease of cell's S/V ratio causes blockage only when the channel size is small. Besides, the elevated membrane stiffness slightly reduces the traverse velocity of the iRBCs, but does not cause blockage in any of our simulations.

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Hierarchical Determination of Nuclear Deformability by Lamin Isoforms during Adult Hematopoiesis: Implications in Blood Cell Trafficking

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Some blood cell types become softer in maturation to facilitate trafficking from marrow through the endothelial barrier and into the circulation. However, any common molecular basis of this phenomenon remains unclear. Cellular deformability is determined by elasticity of the cortex and the nucleus, and the latter is dynamically regulated by changes in expression and organization of the nuclear lamins. We developed a novel protein isoform expression analysis algorithm, ''mass spectrometry calibrated intracellular flow cytometry'', to